Immunohistochemical localisation of vitamin B₁₂ R-binder in the human digestive tract

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SUMMARY The distribution of vitamin B₁₂ R-binder in the human digestive tract was studied using an indirect immunoperoxidase technique. Positive staining for R-binder was found in the mucous cells and ductal epithelial cells of the salivary glands and the oesophageal glands. In normal gastric mucosa, no positive staining for R-binder was found, but in the area with intestinal metaplasia, the columnar epithelial cells and goblet cells showed positive staining. Epithelial cells of the gall bladder, intrahepatic bile ducts and pancreatic ducts were also positive for R-binder. In the small intestine and colon, R-binder was found in the columnar epithelial cells and goblet cells. The measurement of unsaturated vitamin B₁₂ binding capacity and cobalamin content in the extracts from intestinal mucosa also indicated the presence of R-binder in the intestinal mucosa.

R-binder, an immunologically distinct vitamin B₁₂ binding protein, is ubiquitous in human serum, many blood cells and various body fluids. In the digestive tract, R-binder is present in saliva, gastric juice, bile, and pancreatic juice, but its function is still a matter of speculation. Although the localisation of R-binder in the salivary gland is studied in one report,¹ its tissue distribution must be studied in more detail to determine its role.

We studied the distribution of R-binder in the human digestive tract using the immunoperoxidase technique. In addition, we measured unsaturated vitamin B₁₂ binding capacity (UBBC) and endogenous cobalamin in the extracts from the human intestinal mucosa to confirm the results of the immunohistochemical study.

Methods

TISSUE PREPARATION

Specimens of the gastrointestinal tract and the gall bladder were obtained surgically from patients who

had resections for gastric ulcer, gastric cancer, leiomyoma and perforation of the small intestine, colon cancer and cholelithiasis. Specimens of the major salivary gland, liver and pancreas were obtained at necropsy.

Macroscopically normal tissue samples were removed and fixed in 10% formalin and embedded in paraffin. Four micron thick sections were cut and deparaffinised in xylene and ethanol series. For routine histological examination each section was stained with haematoxylin-eosin, and histologically normal sections were used to detect R-binder.

ANTISERUM

R-binder was purified from pooled human saliva using affinity chromatography according to the method of Allen *et al.*^{2,3} Purified R-binder showed a single protein band by SDS-polyacrylamide gel electrophoresis. Anti R-binder antiserum was prepared in rabbits. Specificity of the antiserum was confirmed by Ouchterlony's immunodiffusion method and immunoelectrophoresis.

IMMUNOPEROXIDASE TECHNIQUE

Indirect peroxidase technique was used. Deparaffinised tissue sections were treated with 0.3% hydrogen

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peroxide in methanol for 30 minutes in order to block the endogenous peroxidase activity. Sections were pre-coated with 5% normal goat serum for 20 minutes, and then incubated with anti R-binder antiserum at a dilution of 1:400 with 0.1 M phosphate buffered saline (PBS), pH 7.4, containing 0.5% bovine serum albumin, at 4°C overnight, followed by peroxidase conjugated goat antirabbit IgG antiserum (Cappel, USA) at a dilution of 1:80; at room temperature for 40 minutes. After each application of antiserum, the sections were washed with PBS three times for 10 minutes each time. The histochemical reaction for peroxidase was carried out using 3.3'-Diaminobenzidine (0.05% w/v) and hydrogen peroxide (0.01%) in 0.05 M Tris-HCl buffer, pH 7.6. In control preparations, the primary antiserum was replaced by normal rabbit serum or by specific antiserum preincubated with an excess of purified R-binder: control stainings were all negative.

EXTRACTION OF THE INTESTINAL MUCOSA

Samples of the large and small intestine were obtained from four necropsy cases within two hours after death. In each case, a part of the colon, jejunum and ileum, which showed macroscopically normal mucosa, were removed, washed with saline and frozen immediately at -70°C.

After thawing, the mucosa was scraped off, mixed with an equal volume of 0.1 M potassium phosphate

buffer containing 0.15 M NaCl, pH 7.4, and homogenised for five minutes. The homogenate was centrifuged at 15 000 g for 30 minutes and the supernatant was collected.

ASSAY OF UBBC, TRANSCOBALAMIN II (TcII), ENDOGENOUS COBALAMIN AND PROTEIN CONTENT IN THE EXTRACTS

Unsaturated vitamin B₁₂ binding capacity was measured by the technique of Gottlieb *et al.*,⁴ TcII by absorption to a microfine precipitate of Silica.⁵ Endogenous cobalamin was assayed by vitamin B₁₂ [⁵⁷Co] radioassay (Corning, USA, lot 63015). Protein content was measured using Bio Rad Protein Assay (Bio Rad, USA). UBBC, TcII and cobalamin content in the sera of 10 healthy volunteers were also assayed for comparison.

Results

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In all sections, R-binder positive cells were stained with a clear contrast. Gastrointestinal tract tissues examined contained various numbers of polymorphonuclear leucocytes or histocytes, which served as useful positive controls in judgment.

In the salivary glands, R-binder positive reaction was found in the cytoplasm of mucous cells and intercalated duct cells. Myoepithelial cells and serous

Fig. 1 Immunoperoxidase staining for R-binder in the submandibular gland. Positive staining is found in the mucous cells and intercalated duct cells. Methyl green counterstain.

Fig. 2 Immunoperoxidase staining for R-binder in the gastric mucosa. (a) Normal fundic mucosa, no positive staining is found. (b) Intestinal metaplastic mucosa, positive staining is found in the columnar epithelial cells and goblet cells. (a) and (b), methyl green counterstain.

cells were negative (Fig. 1). Similarly, mucous cells and ductal epithelial cells of the oesophageal glands showed positive staining for R-binder.

In normal gastric mucosa, no positive staining for R-binder was found except for small numbers of

polymorphonuclear leucocytes. In contrast, in the area with intestinal metaplasia, there was always intense staining for R-binder in both columnar epithelial cells and goblet cells (Fig. 2).

Columnar epithelial cells of the gall bladder,

Fig. 3 Immunoperoxidase staining for R-binder in the liver. Positive staining is found in the epithelial cells of intrahepatic bile ducts. Methyl green counterstain.

intrahepatic bile ducts and pancreatic ducts were also positive for R-binder. Hepatocytes and acinar components of the pancreas were negative for R-binder (Figs 3–5).

In the small intestine and colon, R-binder was found in the columnar epithelial cells and goblet cells. The epithelium of the small intestine was stained weakly (Fig. 6).

UBBC TCII AND COBALAMIN CONTENT IN THE EXTRACT

The Table shows the UBBC, TcII and cobalamin content in the extracts of each case. In all cases there was detectable UBBC and TcII. The level of total vitamin B₁₂ binding capacity (UBBC+cobalamin) in all extracts was clearly higher than that in the serums. The colonic extracts showed higher levels of UBBC than those of the small intestine in all cases, and there was no difference between the levels of ileum and jejunum.

Discussion

Our immunohistochemical study revealed that R-binder was present in almost all digestive organs. In the oral cavity and the oesophagus, saliva and mucus of the oesophagus contain R-binder secreted from the salivary glands and oesophageal glands. Nexø *et al*¹ showed, using an immunohistochemical tech-

nique, that R-binder was present both in the mucous secretory acini and in the intercalated ducts of the human salivary glands. Although they used anti-hog R-binder antiserum as the primary antiserum, our findings obtained using antihuman R-binder antiserum were consistent with theirs.

In normal gastric mucosa, R-binder was not found in the surface epithelium or in the secretory glands, but in the area with intestinal metaplasia, the columnar epithelial cells and goblet cells showed positive staining for R-binder. Allen *et al*⁶ reported that gastric juice of 11 individuals had ranged from 89–100% in terms of percentage of inhibition of vitamin B₁₂ binding activity by anti-intrinsic factor antibody. Marcoullis *et al*⁷ reported that extracts of human stomach mucosa contained immunoreactive intrinsic factor and small amounts of R-protein and TcII, which was possibly a contaminant from the blood. Thus R-binder in the stomach seems to be in rather small amounts, if any. R-binder might not be present in normal gastric mucosa and the main source of R-binder in gastric juice might be derived from swallowed saliva or from secretions of intestinal metaplastic mucosa which is found frequently in adults.

Our results also suggest that the source of R-binder in bile and pancreatic juice is from epithelial cells of the gall bladder, bile ducts and pancreatic ducts. R-binder was present in the epithelium of the small and

Fig. 4 Immunoperoxidase staining for R-binder in the gall bladder. Positive staining is found in the epithelial cells of the gall bladder. Methyl green counterstain.

large intestine. TcII is reported^{*} to be present in the intestine, whereas the R-binder has not yet been found to be present in the small or large intestinal mucosa. To confirm these findings, we measured UBBC and cobalamin content in the extracts from

human intestinal mucosa. The results clearly showed the presence of R-binder in human intestinal mucosa even if there may be small amounts of contamination of blood or R-binder derived from blood cells in the mucosa. Staining grades in the immunohistochemical

Fig. 5 Immunoperoxidase staining for R-binder in the pancreas. Positive staining is found in the epithelial cells of the pancreatic ducts. Methyl green counterstain.

Fig. 6 Immunoperoxidase staining for R-binder in the intestine. (a) Small intestine, weakly positive staining is found in the columnar epithelial cells and goblet cells. (b) Large intestine, positive staining is found in the columnar epithelial cells and goblet cells. (a) and (b), Methyl green counterstain.

study, which showed more intense staining for R-binder in the colon than in the small intestine, were paralleled with the data that UBBC and content of cobalamin in the extracts from the colon were always higher than those from the small intestine. In the intestinal metaplastic tissue R-binder was stained

heavily in comparison with normal small intestinal tissue. It is speculated that there may be larger amounts of R-binder in the intestinal metaplastic tissue than in normal small intestinal tissue, however the reason is uncertain.

A previous study⁹ indicated, using the gel chroma-

Table Unsaturated vitamin B₁₂ binding capacity (UBBC), TcII and cobalamin content in the extracts from the human intestine and in the sera

Case	Age	Sex	Disease	Material	Value (pg/mg-protein)		
					Cobalamin	UBBC	TcII
1	42	Male	renal cancer	Colon	88.5	122.6	3.9
				Ileum	62.2	11.1	2.9
2	64	Female	uterus cancer	Jejunum	79.0	10.0	0.8
				Colon	988.7	235.3	4.1
3	54	Female	uterus cancer	Ileum	696.4	29.1	3.7
				Jejunum	747.7	31.0	2.7
4	83	Male	myocardial infarction	Colon	873.7	40.3	2.1
				Ileum	581.1	13.4	1.3
				Jejunum	182.9	8.2	1.6
				Colon	280.7	94.5	9.6
				Ileum	291.0	4.5	2.7
				Jejunum	137.5	8.4	4.6
Normal volunteer (n=10)				Serum	7.7±1.6	15.9±4.5	9.3±1.6

Values in serum are expressed as mean ± 1 SD. TcII=transcobalamin II.

tographic technique for aspirated human intestinal fluid, that R-binder of unknown origin was present in the intestinal lumen. Our results suggest that intestinal epithelial cells secrete R-binder into the lumen. The role of R-binder remains uncertain, but it is suggested that R-binder excretes so called cobalamin analogues from the human body, and inhibits bacterial overgrowth.¹⁰⁻¹² Intestinal R-binder may protect the overgrowth of intestinal bacterial flora, and at the same time it may protect the entrance of non-physiological cobalamin analogues produced by intestinal microorganisms.^{13,14}

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